REVIEW ARTICLE

Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human microbiome

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Abstract

Water is one of the most important bacterial habitats on Earth. As such, water represents also a major way of dissemination of bacteria between different environmental compartments. Human activities led to the creation of the so-called urban water cycle, comprising different sectors (waste, surface, drinking water), among which bacteria can hypothetically be exchanged. Therefore, bacteria can be mobilized between unclean water habitats (e.g. wastewater) and clean or pristine water environments (e.g. disinfected and spring drinking water) and eventually reach humans. In addition, bacteria can also transfer mobile genetic elements between different water types, other environments (e.g. soil) and humans. These processes may involve antibiotic resistant bacteria and antibiotic resistance genes. In this review, the hypothesis that some bacteria may share different water compartments and be also hosted by humans is discussed based on the comparison of the bacterial diversity in different types of water and with the human-associated microbiome. The role of such bacteria as potential disseminators of antibiotic resistance and the inference that currently only a small fraction of the clinically relevant antibiotic resistome may be known is discussed.

Introduction

The development and spread of antibiotic resistance among bacteria is considered a universal threat to human, animal and environmental health. Numerous studies have demonstrated the importance of the environmental settings (e.g. water or soil) on the cycling of antibiotic resistance in nature, either because antibiotic resistance mechanisms can originate in environmental bacteria or because human and animal commensals and pathogens can contaminate the environment (Allen *et al.*, 2010; Baquero *et al.*, 2008; Martinez, 2008; Riesenfeld *et al.*, 2004; Zhang *et al.*, 2009).

Water is one of the most important bacterial habitats on Earth, is a major way of dissemination of microorganisms in nature and has been recognized as a significant reservoir of antibiotic resistance (Baquero *et al.*, 2008; Rizzo *et al.*, 2013; Zhang *et al.*, 2009). As a microbial habitat, water may represent the origin of resistance genes, be an amplifier and/or reservoir of genes already acquired by human pathogens and released as pollutants in the environment or act as a bioreactor, facilitating the interchange of resistance genes between pathogenic and nonpathogenic bacteria (Baquero *et al.*, 2008; Poirel *et al.*, 2005; Rizzo *et al.*, 2013). However, and in spite of the intense research in this area over the last years, it is not clear under which circumstances water bacteria are important sources of novel mechanisms of antibiotic resistance or when do they act as carriers or helper elements that, somehow, facilitate the spread of antibiotic resistance.

Another question, still unanswered, regards the modes by which antibiotic resistance in water may be relevant for human health. Because antibiotic resistance is harbored and transferred by bacteria, a better understanding of the bacterial diversity and ecology may bring interesting insights into the modes of resistance dissemination from and into humans. This approach is now possible because numerous studies conducted worldwide have explored the bacterial diversity in water habitats over the

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last decades. In parallel, the human microbiome project has stimulated the thorough characterization of the diversity of bacteria that permanently or transiently can colonize the human body. The combination of both datasets may bring interesting information for the discussion of antibiotic resistance transmission from water to humans and vice versa.

This work discusses the hypothesis that bacteria sharing different water compartments and also the human body may represent important pieces in the network of antibiotic resistance dissemination. In addition, the cross-comparison of the bacterial diversity in human and water habitats vs. the currently identified antibiotic resistance genes is used to sustain the hypothesis that an important fraction of the clinically relevant antibiotic resistome may be yet to be unveiled.

The urban water cycle

Over the centuries, humans settled their lives preferentially in sites around water reservoirs, creating high population densities in these areas and also major sources of pollution. The implementation of sanitation processes capable of removing contaminants (chemical pollutants, organic matter, microorganisms) from wastewater before its discharge into the natural environment became a priority. In the same way, the supplying of clean and safe drinking water, often requiring purification and disinfection, is nowadays regarded as a basic human right, essential for an effective policy for health protection (WHO & UNICEF, 2000). Throughout the years, the scientific knowledge and numerous technologic advances contributed to the continuous improvement of processes for the provision of safe water and appropriate disposal and treatment of wastewater. These two stages constitute the man-made or urban water cycle.

Bacterial diversity in water habitats

Freshwater habitats are amongst the natural habitats that harbor the richest bacterial diversity (Tamames *et al.*, 2010). In a comparative study involving 16S rRNA gene sequences from 3502 sampling experiments of natural and artificial bacterial habitats, Tamames *et al.* (2010) concluded that soil and freshwater, represented by aquifers, groundwater, lakes, rivers, drinking water and wastewater, are the natural habitats that harbor the largest number and most diverse group of bacterial lineages. In this study, the bacterial diversity in different freshwater habitats within the urban water cycle was compared (Fig. 1 and Supporting Information, Table S1). This comparison was based on studies published after 1995 in journals indexed to the ISI – Web of Knowledge, in which the major objective was the analysis of the water bacterial diversity, supported by 16S rRNA gene sequence analysis.

At high taxonomic ranks of phylum or class, in general, the most predominant bacteria belong to the phyla *Proteobacteria* (mainly of the classes *Alpha-*, *Beta-* and *Gammaproteobacteria*), *Actinobacteria*, *Bacteroidetes* and *Firmicutes*, irrespective of the type of water surface (lakes, rivers, wetlands), mineral, drinking and wastewater (Fig. 1, Table S1). However, different types of water present distinct patterns of bacterial diversity at lower taxonomic ranks, of genus or species. At least this was the conclusion drawn whenever, according to the publications supporting this comparison, the 16S rRNA gene sequence analysis allowed such a discrimination. An apparent specificity for some types of water was observed. For example, members of the class *Betaproteobacteria* and of the phylum *Bacteroidetes* were frequently detected in surface, mineral and drinking water, but not so often in wastewater. In turn, *Firmicutes* were frequently reported in wastewater. Ubiquitous bacteria are those with low specificity, occurring in different environments, including throughout the urban water cycle or in the interface air-water-soil (Tamames *et al.*, 2010; Fig. 1 and Table S1). At the genus rank, examples of the most ubiquitous bacteria in water habitats, that is those detected in wastewater, surface- and drinking water, are members of the genera *Acidovorax*, *Curvibacter*, *Sphingomonas*, *Aeromonas*, *Acinetobacter*, *Pseudomonas*, *Legionella*, *Rhodococcus*, *Gordonia*, *Mycobacterium*, *Flavobacterium*, *Bacillus* and *Clostridium* (Fig. 1 and Table S1). Bacteria belonging to these groups, and others still unidentified, are probably capable of circulating between different aquatic habitats, spanning the whole urban water cycle.

The use of culture-independent approaches, mainly the high throughput sequencing methods, brought a renewed perspective of the bacterial diversity in water habitats, in which < 0.1% of bacteria can be cultivated (Amann *et al.*, 1995; Simon & Daniel, 2011; Vaz-Moreira *et al.*, 2013). These approaches revealed that bacteria still unidentified below the phylum or class levels are detected in every type of water (Table S1). This is particularly notorious for some bacterial phyla/classes, which despite the apparent poor culturability are common water inhabitants. Good examples of groups almost or exclusively detected by culture-independent methods are members of *Delta-* and *Epsilonproteobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Cyanobacteria*, *Nitrospirae*, *Planctomycetes*, *Chloroflexi*, *Chlorobi*, *Gemmatimonadetes*, *Spirochaetes*, *Chlamydiae*, *Aquificae*, *Thermotogae*, *Fusobacteria*, *Synergistetes* and *Tenericutes*, some of them including bacteria ubiquitous in water habitats (Fig. 1, Table S1). Nevertheless, culture-independent methods, even high throughput sequencing,

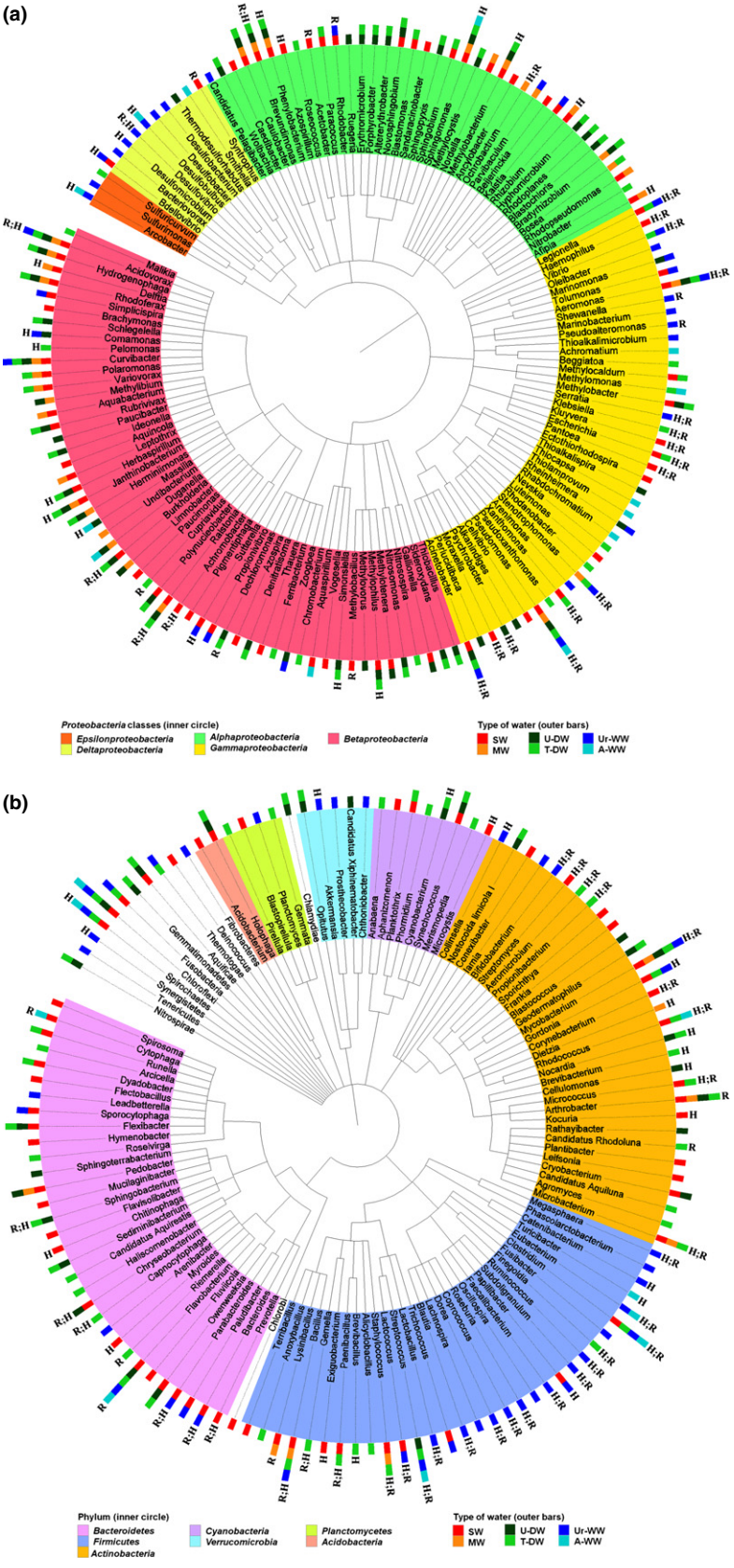

Fig. 1. Dendrogram representations of the bacterial diversity [(a) *Proteobacteria* classes and (b) other phyla] observed in different types of water, occurrence in the human-associated microbiome (H) and previous description of antibiotic resistance genes (R). The dendrograms were constructed with the iTOL – interactive tree of life (Letunic & Bork, 2007, 2011), based on the taxon ID codes, corresponding to the identifications provided in each of the publications cited (see Table S1). Different phyla or *Proteobacteria* classes (inner circle) are represented by different colors (when are represented by two or more bacterial genera), and the presence in different types of water are represented by the outer bars. Types of water: SW, surface water that includes W (wetlands), R (rivers), L (lakes); MW, mineral drinking water that also includes spring water; U-DW, untreated drinking water; T-DW, treated drinking water; Ur-WW, urban domestic wastewater that may also include industrial wastewaters; A-WW, animal wastewater.

may fail on the detection of some bacterial groups, in particular the less abundant organisms (Pinto & Raskin, 2012). Different biases (e.g. DNA extraction, PCR or sequence data analysis) may hamper the detection of certain community members. On the other hand, the 16S rRNA gene sequence analysis, particularly of small gene fragments as those generated with high throughput sequencing methods, may not allow a reliable identification of bacteria (e.g. Clarridge, 2004). These arguments may explain why bacteria of the genera *Escherichia* or *Enterococcus*, used as indicators of fecal contamination, and frequently detected in wastewater habitats at counts as high as 10^4–10^6 colony-forming units per mL (Ferreira da Silva *et al.*, 2007; Garcia-Armisen & Servais, 2004; Levantesi *et al.*, 2010) are not detected in studies surveying the bacterial diversity, as those summarized in Fig. 1. The low abundance of these bacteria in water habitats, even in those with fecal contamination, is also suggested by cultivation procedures. Indeed, the cultivation of *Escherichia* or *Enterococcus* usually requires the use of selective culture media, while on general culture media, such as Plate Count Agar, if isolated, they represent a small fraction of the cultivable populations. Although both approaches are truly complementary to explore the bacterial diversity of an ecosystem, the current state of the art suggests a poor synchronization between culture-independent and culture-dependent methods. This represents a serious limitation in a comprehensive analysis of the bacterial diversity, mainly when the assessment of the features such as metabolism, physiology, genetics, virulence and antibiotic resistance of a specific group is under discussion. Expectably, one of the major outcomes of the implementation of culture-independent methods will be the improvement of cultivation methods and the strengthening of studies based on pure cultures (Anonymous, 2013; Lagier *et al.*, 2012; Prakash *et al.*, 2013). These advances will be indispensable to the thorough assessment of possible intersections between distinct microbiomes, for example, environmental and human.

Evidences of the natural antibiotic resistome

Over the last 70 years, clinically relevant antibiotic resistance, that is in pathogens and opportunistic bacteria, increased to worrisome levels, mainly in areas with strong human intervention (Andersson & Hughes, 2011; Baquero *et al.*, 2008; Cantón & Morosini, 2011; Martinez, 2009). Nevertheless, antibiotic resistance is a natural property of bacteria, occurring in environments with reduced or null anthropogenic impacts, such as wild life or remote Earth zones (Allen *et al.*, 2010; D'Costa *et al.*, 2006, 2011; Dantas *et al.*, 2008; Riesenfeld *et al.*, 2004; Segawa *et al.*, 2013). In part this can be due to the fact that antibiotics production is ancient in nature, with more than 10^6–10^9 years (D'Costa *et al.*, 2011). Functions, as diverse as molecular signaling, transcription activation, enhanced gene transfer, stimulation of bacterial adhesion, increased mutation frequency or virulence suppression, have been attributed to antibiotics produced in nature (Dantas *et al.*, 2008; Davies *et al.*, 2006; Sengupta *et al.*, 2013; Wright, 2007). Eventually these functions will vary among the target bacteria and will depend on the genetic and physiological environment of the cell. Accordingly, natural antibiotic resistance mechanisms are those that make these molecules compatible with the normal cell function (Sengupta *et al.*, 2013; Wright, 2007). Natural antibiotic resistance has been studied in depth in soil bacteria of the phyla *Actinobacteria*, *Proteobacteria*, or *Bacteroidetes*, mainly in those yielding antibiotic production or degradation activity (D'Costa *et al.*, 2006, 2011; Dantas *et al.*, 2008; Forsberg *et al.*, 2012; Riesenfeld *et al.*, 2004). However, natural antibiotic resistance is not restricted to soil bacteria, being also reported in other environments, including water.

Mineral and spring waters are good examples of natural water habitats, since these aquifers originate in ground water sources and are protected from human intervention (European Comission, 2009; Rosenberg, 2003). Unlike tap water, mineral and spring water cannot be disinfected by any kind of treatment to remove or destroy microorganisms (European Comission, 2009) and, thus, its microbiota mirrors the natural populations of the aquifer. Because this type of water is known to contain a rich microbiota and it is destined to human consumption, several studies have searched the presence of antibiotic resistant bacteria (Falcone-Dias *et al.*, 2012; Mary *et al.*, 2000; Massa *et al.*, 1995; Messi *et al.*, 2005; Rosenberg & Duquino, 1989; Zeenat *et al.*, 2009). Although in some of these studies the experiments were not designed to survey bacterial diversity and antibiotic resistance, it is possible to infer about the wide diversity of antibiotic resistance patterns and the frequent occurrence of multi-resistance phenotypes. Mineral or spring bottled waters commercialized in Italy, Portugal, France and other world regions contained bacteria resistant to multiple antibiotics, distributed by several genera and species (*Afipia*, *Bosea*, *Brevundimonas*, *Ochrobactrum*, *Curvibacter*, *Ralstonia*, *Variovorax*, *Acinetobacter*, *Klebsiella*, *Moraxella*, *Pseudomonas*, *Flavobacterium*, *Pedobacter*, *Arthrobacter*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Bacillus*, *Kurthia*, and *Staphylococcus*) (Massa *et al.*, 1995; Mary *et al.*, 2000; Messi *et al.*, 2005; Zeenat *et al.*, 2009; Falcone-Dias *et al.*, 2012). Bottled spring water bacteria can reach densities as high as 10^2 colony-forming units per mL and display resistance to more than 20 antibiotics belonging to eight different classes, including 3rd generation cephalosporins,

carbapenems and fluoroquinolones (Falcone-Dias *et al.*, 2012). It is remarkable that, in general, studies conducted in different geographic areas and in different occasions demonstrate that the natural microbiota of mineral and spring waters contains a myriad of antibiotic resistant bacteria, as was observed before for pristine soils or ancient permafrost samples (e.g. Allen *et al.*, 2009; D'Costa *et al.*, 2006, 2011). Many of these (multi-)drug resistance phenotypes are probably intrinsic in these bacteria, and resistance transfer to human-related bacteria can be considered highly unlikely. These considerations require a further discussion about the nature of the environmental antibiotic resistome.

Acquired, intrinsic and silent resistance: different assets in the same game

Most of the discussions on antibiotic resistance are centered on acquired resistance, resultant from gene mutation or genetic recombination by horizontal gene transfer (conjugation, transformation or transduction) (Martinez & Baquero, 2000; Livermore, 2003; Tenover, 2006; Zhang *et al.*, 2009; Davies & Davies, 2010). Although these can be random processes, in the presence of selective pressures, such as antimicrobial residues, bacterial lineages with acquired antibiotic resistance will have an improved fitness (i.e. a better capacity to survive and reproduce in comparison with bacteria without acquired resistance), becoming more prevalent in the community (Andersson & Hughes, 2011; Barbosa & Levy, 2000; Martinez, 2009).

In contrast, the intrinsic resistome is described as an ensemble of nonacquired genes with influence on the susceptibility to antibiotics (Baquero *et al.*, 2013; Fajardo *et al.*, 2008). This form of resistance comprises diverse mechanisms that can be related with structural, physiological or biochemical properties of bacteria, such as reduced permeability, metabolic functions, efflux systems, among others (Alvarez-Ortega *et al.*, 2011; Baquero *et al.*, 2013; Fajardo *et al.*, 2008; Martinez, 2008; Wright, 2010). Intrinsic antibiotic resistance represents a characteristic phenotype of a species or organism, resultant from multiple genes and, hence, is not easily transferable by horizontal gene transfer. In the same way, it is not the direct consequence of adaptation to antibiotics (Alvarez-Ortega *et al.*, 2011).

Since about 3% of the genes in a bacterial genome may be related with intrinsic resistance processes (Fajardo *et al.*, 2008), it is expected that this native resistance form represents an important fraction of the environmental antibiotic resistome. A well characterized intrinsic resistome belongs to the opportunistic pathogen *Pseudomonas aeruginosa*, which displays intrinsic resistance to a wide variety of antibiotics, resultant from a complex network of genes (Alvarez-Ortega *et al.*, 2011; Breidenstein *et al.*, 2011; Fajardo *et al.*, 2008). The low permeability of the external membrane, 12–100 times lower in *P. aeruginosa* than in *E. coli*, and the presence of some proteins involved in the alteration of cell metabolism, leading, for instance, to changes in the cell growth state, are supposed to represent the most important mechanisms of intrinsic resistance in this organism (Hancock, 1998; Hancock & Brinkman, 2002; Alvarez-Ortega *et al.*, 2011; Breidenstein *et al.*, 2011).

Although intrinsic resistance may be characteristic of a species, it is not necessarily common to all species members. In *E. coli*, point mutations in different *loci* were observed to promote reduced susceptibility to antibiotics such as ciprofloxacin, rifampin, vancomycin, ampicillin, sulfamethoxazole, gentamicin, or metronidazole (Tamae *et al.*, 2008). The potential of some members of a species to mutate towards significant reduction or increase in antibiotic susceptibility was observed in different species (e.g. *Helicobacter pylori*, *Acinetobacter baylyi*, *P. aeruginosa*), being probably species-specific (Fajardo *et al.*, 2008; Girgis *et al.*, 2009; Gomez & Neyfakh, 2006; Liu *et al.*, 2010). This kind of genome variations in bacterial populations is probably common in nature and may have interesting implications on the ecology of antibiotic resistant bacteria.

The implications of the intrinsic resistome on the evolution of acquired antibiotic resistance are not completely understood. However, the characterization of the intrinsic resistome genes may bring important contributes to predict the stability, emergence and evolution of antibiotic resistance (Fajardo *et al.*, 2008; Martinez *et al.*, 2007). In a community, it is possible that intrinsic resistance will drive bacterial selection, leading to community rearrangements, mainly when selective pressures, as those imposed by antibiotics, are present (Baquero *et al.*, 2013). Hypothetically, if a bacterial population is intrinsically resistant, it will have higher chances to survive in the presence of antimicrobial residues, and to get in contact with potential resistance donors, proliferating more and faster than nonintrinsically resistant organisms. Thus, it can be hypothesized that intrinsic resistance, at least in some highly ubiquitous bacteria, may represent an advantage for resistance acquisition. A good example of how intrinsic resistance may favor resistance acquisition may be represented by *P. aeruginosa*, one of the opportunistic pathogens with highest potential to acquire antibiotic resistance (Breidenstein *et al.*, 2011).

A major question may be whether genes related with intrinsic resistance phenotypes may be transferred horizontally. Although such an event is not supposed to occur, at least at a high frequency, conceivably, it is not impossible. Other resistance determinants not included in

the classical antibiotic-resistance genes, may also occur in nature, and bring interesting insights into the ecology of antibiotic resistance. Silent resistance genes are hidden forms of antibiotic resistance that do not confer resistance to its native host, although are capable of conferring resistance when expressed in other hosts (Dantas & Sommer, 2012).

In summary, the natural antibiotic resistome comprises three categories: (1) those designated as acquired resistance genes, which correspond to the classical antibiotic-resistance genes, (2) the genes related with intrinsic resistance and (3) the silent resistance genes. Because some of these genes may respond to unspecific stimuli, and not only to antibiotics, they may contribute to the selection of the antibiotic unsusceptible populations (Baquero *et al.*, 2013; Dantas & Sommer, 2012). These arguments reinforce the need to study antibiotic resistance in a global perspective either in the context of the cell genome or the whole bacterial community.

Antibiotic resistance in wastewater

Among the man-made environments, wastewater treatment plants (WWTP) are the most important receptors and suppliers of human derived antibiotic resistance (Manaia *et al.*, 2012; Rizzo *et al.*, 2013). The indicators of fecal contamination, *E. coli* and *Enterococcus* spp., are often used to monitor antibiotic resistance prevalence in urban wastewaters (Ur-WW). In these groups, high resistance prevalence values have been observed for antibiotics with a long history of use, such as aminopenicillins, sulfonamides and tetracyclines for *E. coli* or tetracycline and erythromycin for enterococci (Manaia *et al.*, 2012). Moreover, it is shown that conventional wastewater treatment does not contribute to reduce the fraction of antibiotic resistant bacteria, leading, sometimes, to its increase in the final effluent (Ferreira da Silva *et al.*, 2006, 2007; Łuczkiewicz *et al.*, 2010; Novo *et al.*, 2013). It is impressive that in different world regions and using distinct types of wastewater treatment, WWTP are responsible for the discharge of about one billion of culturable antibiotic resistant coliforms per minute to the environment (exemplified for ciprofloxacin resistance in Fig. 2). Despite the relevance of *E. coli* and *Enterococcus* as indicators of human fecal contamination, apparently these bacteria are not the most prevalent bacterial groups in sewage sludge or in wastewater (Sanapareddy *et al.*, 2009; McLellan *et al.*, 2010; Xia *et al.*, 2010b; Yang *et al.*, 2011; Wang *et al.*, 2012; Ye & Zhang, 2012; Zhang *et al.*, 2012) (Fig. 1). Indeed, *E. coli* and enterococci are probably minor representatives of the water bacterial communities. This conclusion leads us to a new dilemma. If most of the well-known bacteria in terms of antibiotic resistance are minor representatives of wastewater communities, it is reasonable to argue that other community members, mainly the most abundant, may play also important roles as donors, receptors or simply mediators of antibiotic resistance dissemination.

Antibiotic resistance in aquaculture environments

In aquaculture, antimicrobials are routinely used through the direct addition into the water body. However, the

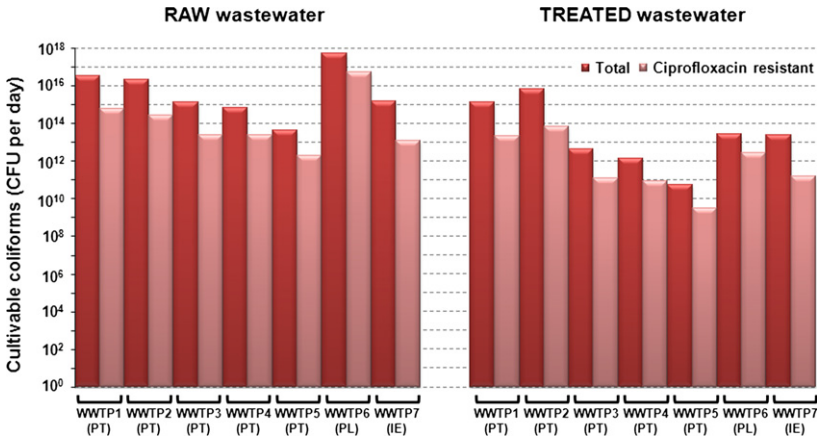

Fig. 2. A domestic wastewater treatment plant (WWTP) discharges about 1 billion (10^9) ciprofloxacin resistant coliforms per minute. Total and ciprofloxacin resistant coliforms (CFU per day) discharged by WWTP in different countries [WWTP1-WWTP5, Portugal (PT); WWTP6, Poland (PL); WWTP7, Ireland (IE)], with different sizes (average day flow of 20 000, 32 500, 900, 890, 200, 96 000 and 49 000 m^3, respectively) and treatment processes [activated sludge (WWTP1 and WWTP6), trickling filter (WWTP2), submerged aerated filter (WWTP3), aeration lagoon (WWTP4), anaerobic lagoon (WWTP5), unknown secondary treatment (WWTP7), with bacterial removal rates above of 1.5–4 log (CFU; Galvin *et al.*, 2010; Łuczkiewicz *et al.*, 2010; Manaia *et al.*, 2010; Novo & Manaia, 2010).

negative impacts of this procedure have been demonstrated and include the persistence of antimicrobial residues in water and fish and the selection and spread of resistance genes, with the consequent contamination of the environment and the human food-chain (Sørum, 1998; Cabello, 2006; Taylor *et al.*, 2011; Tamminen *et al.*, 2011). The spread of antibiotic resistance among fish pathogens has economic impacts on aquaculture productivity and increases the possibilities of the dissemination of resistance determinants to other bacteria, including human pathogens (Cabello, 2006; Rhodes *et al.*, 2000). The long term effects are demonstrated by the fact that, even in the absence of selective pressures, when the antibiotic used was banned from an aquaculture system, genes conferring low susceptibility to that antibiotic will persist (Tamminen *et al.*, 2011). Bacterial diversity studies in aquaculture water bodies are scant, but the presence of some genera, such as *Yersinia*, *Vibrio*, *Photobacterium*, *Pseudomonas* and *Aeromonas,* is consistently reported (Ozaktas *et al.*, 2012; Rodríguez-Blanco *et al.*, 2012; Schulze *et al.*, 2006; Sørum, 1998). These genera comprise also some bacteria with important roles on antibiotic resistance spread, for example *qnr*A, encoding a DNA topoisomerase protector and the extended-spectrum beta-lactamase PER-6 (Girlich *et al.*, 2010a; Poirel *et al.*, 2005). Moreover, the dissemination of antimicrobial resistance in aquaculture environments may be associated with other resistance determinants such as heavy metals or biocides (Akinbowale *et al.*, 2007; Cabello *et al.*, 2013; Rodríguez-Blanco *et al.*, 2012; Seiler & Berendonk, 2012).

Antibiotic resistance in disinfected drinking water

Despite the scarce information regarding antibiotic resistance in disinfected drinking water, it was already demonstrated that it may contain bacteria, such as those of the genera *Sphingobium*, *Sphingomonas*, *Pseudomonas* and *Acinetobacter* or nonfecal *Enterobacteriaceae* capable of resisting different antibiotics (Faria *et al.*, 2009; Xi *et al.*, 2009; Vaz-Moreira *et al.*, 2011b, 2012; Figueira *et al.*, 2012; Narciso-da-Rocha *et al.*, 2013) (Table S2). For instance, *Sphingomonadaceae*, a bacterial group recognizedly ubiquitous, rich in mobile genetic elements, and comprising common inhabitants of environments contaminated with xenobiotcs, can be highly prevalent in disinfected drinking water (Koskinen *et al.*, 2000; Furuhata *et al.*, 2007; Stolz, 2009; Aylward *et al.*, 2013). Tap water *Sphingomonadaceae* yield a rich and diversified resistance pattern to penicillins, cephalosporins, carbapenems and aminoglycosides (Vaz-Moreira *et al.*, 2011b), but their relevance on the spread of antibiotic resistance is unknown.

Independent studies have demonstrated that antibiotic resistant bacteria, at least for some classes of antibiotics, may be more prevalent in tap than in the water source (Gomez-Alvarez *et al.*, 2012; Narciso-da-Rocha *et al.*, 2013; Vaz-Moreira *et al.*, 2012; Xi *et al.*, 2009). Such an effect may be due either to the selective effect of the disinfection processes or to the income of antibiotic resistant bacteria downstream the disinfection point (Gomez-Alvarez *et al.*, 2012; Vaz-Moreira *et al.*, 2013). This is a fundamental and difficult to answer question, given the complex rearrangements in the bacterial communities that result from the disinfection processes (Eichler *et al.*, 2006; Figueira *et al.*, 2011; Hoefel *et al.*, 2005; Kormas *et al.*, 2010; Vaz-Moreira *et al.*, 2013). However, strain tracking approaches do not support the conclusion that the water source is the most probable origin of the antibiotic resistance detected in tap water (Narciso-da-Rocha *et al.*, 2013; Vaz-Moreira *et al.*, 2011b, 2012). Regarding the origin of the antibiotic resistance found in drinking water, it has been observed that the majority of the resistance phenotypes in bacteria of groups such as *Sphingomonadaceae*, *Pseudomonas* or *Acinetobacter* is species dependent. This observation suggests a pattern of vertical inheritance of resistance and, thus, it can be hypothesized that antibiotic resistance in these organisms is probably intrinsic (Narciso-da-Rocha *et al.*, 2013; Shehabi *et al.*, 2005; Vaz-Moreira *et al.*, 2011b, 2012). Either being acquired or intrinsic, the impacts that antibiotic resistant bacteria present in drinking water may have on human health are still unknown.

Antibiotic resistance genes throughout the urban water cycle

The tracking of antibiotic resistance genes in different environmental compartments is an important tool to assess the ecology and epidemiology of antibiotic resistance. Antibiotic resistance genes, encoding every known type of mechanism (target protection, target modification, drug modification, reduced permeability or efflux), are found throughout the urban water cycle (Table S2). These genes have been detected either in bacterial isolates or in total genomic DNA samples, using, most of the times, primers or probes targeting antibiotic resistance genes that are already known. Most of such primers and probes were designed based on genome sequences of bacterial isolates yielding a given resistance phenotype. Therefore, the vast majority of surveys of antibiotic resistance genes rely, directly or indirectly, on cultivable bacteria recognized as opportunists or pathogens. Examples of the most common hosts of the well-known antibiotic resistance genes are members of the family *Enterobacteriaceae* (e.g. genera *Klebsiella*, *Citrobacter*, *Enterobacter*, *Raoultella*) or

the genera *Acinetobacter*, *Aeromonas*, *Burkholderia*, *Pseudomonas*, *Enterococcus*, *Staphylococcus* and some other that in total represent a humble fraction of the bacterial groups thriving in water habitats.

Wastewater, in particular raw, is the richest water habitat in known antibiotic resistance genes. There, can be found a typical signature of genes encoding resistance to 'old' antibiotics such as tetracyclines, sulfonamides, aminoglycosides and beta-lactams (e.g. *tet*, *aac*, *dfr*, *sul*, class A beta-lactamases; Table S2). Most of these genes are located in plasmids and some are part of the variable gene cassettes of integrons and, probably, can easily be mobilized amongst bacteria (Garcillán-Barcia *et al.*, 2011; Partridge, 2011). Recently, Zhang *et al.* (2011) demonstrated that plasmids, mainly harbored by *Proteobacteria* of the classes *Alpha-*, *Beta-* and *Gamma-* and members of the genera *Bacillus*, *Mycobacterium* and *Nocardiopsis*, some of which are abundant in wastewater habitats, are relevant vectors of tetracycline, macrolide and multidrug resistance genes in these environmental niches.

Studies reporting the diversity and abundance of antibiotic resistance genes in drinking water are scarce. However, the occurrence of genes also detected in clinical isolates, encoding resistance to beta-lactams, aminoglycosides, macrolides or sulfonamides is described even in disinfected water (Table S2) (Faria *et al.*, 2009; Xi *et al.*, 2009; Figueira *et al.*, 2012). The origin of these resistance genes in drinking water is still unknown, being unclear in which cases it results from environmental contamination. A major limitation to answer this question is related with the fact that most of the drinking water bacteria are of environmental origin and poorly or not at all characterized in terms of antibiotic resistance genes (Fig. 1, Table S1).

Commonly used arguments to explain the evolutionary success of acquired antibiotic resistance

Acquired antibiotic resistance is an emblematic example of biological evolution, driven by two major mechanisms – genetic variability (mutation and recombination) and selection (Andersson & Hughes, 2010; Thomas & Nielsen, 2005; Wiedenbeck & Cohan, 2011). Genetic variability results from gene mutation and horizontal gene transfer, in which the latter has more dramatic implications on the physiology and ecology of bacteria (Arber, 2000; Hausner & Wuertz, 1999; Miyahara *et al.*, 2011). On the other hand, antibiotics, even at subinhibitory concentrations, or other micro-pollutants such as heavy metals, contribute for the selection of resistant bacteria (Alonso *et al.*, 2001; Davies *et al.*, 2006; Tello *et al.*, 2012). However, the selection of antibiotic resistant bacteria may not represent the only consequence of the environmental contamination with antibiotics. Actually, the residues of antibiotics at environmental concentrations (often subinhibitory) are also correlated with disturbances on the structure and composition of bacterial communities in water habitats (Huerta *et al.*, 2013; Novo *et al.*, 2013). Moreover, in the environment, pollutants occur in complex mixtures, which make it difficult to predict their effects on the microbial communities. Processes of co- or cross-resistance, for instance, due to genetic linkage or to broad enzyme specificity, may lead to the selection of resistance genes in the absence of a selective pressure by antibiotics (Baker-Austin *et al.*, 2006; Harada & Asai, 2010). If the above mentioned arguments could explain antibiotic resistance proliferation, acquired antibiotic resistance would be detected only in habitats such as wastewater or in the animal or human body, mainly in the gut, during antibiotherapy periods. However, this is not the case and antibiotic resistance determinants are found in environments where none of the above mentioned pressures are present (Harada & Asai, 2010). The strongest argument to explain the occurrence of recognized clinically relevant resistance genes in environments with no apparent selective pressure refers to the low fitness costs of antibiotic resistance genes (i.e. when antibiotic resistance acquisition do not reduce the survival and proliferation of a bacterium, even in the absence of selective pressures) (Andersson & Hughes, 2010; Gullberg *et al.*, 2011). The influence of compensatory mutations on the reduction of fitness costs imposed by acquired antibiotic resistance has been demonstrated (Andersson & Hughes, 2010; Björkman *et al.*, 2000; Handel *et al.*, 2006; Maisnier-Patin & Andersson, 2004; Schulz zur Wiesch *et al.*, 2010; Tanaka & Valckenborgh, 2011). Since compensatory mutations may alleviate the fitness costs associated with a given acquired resistance, resistant and susceptible bacteria will display a comparable fitness in the environment, although with different levels of tolerance to antibiotics. As a consequence, strains harboring resistance and compensatory mutations may have a selective advantage in the environment, mainly in the presence of antimicrobial residues (Andersson & Hughes, 2010; Björkman *et al.*, 2000; Handel *et al.*, 2006; Schulz zur Wiesch *et al.*, 2010). The importance of the environmental conditions on the selection of resistance and compensatory mutations is suggested by the fact that different fitness-compensating mutations are observed in bacteria thriving in mice or in a laboratory medium (Björkman *et al.*, 2000). These evidences emphasize the complexity of the antibiotic resistance ecology, although it seems reasonable to assume that as long as bacteria and/or genetic elements are able to move across different water habitats, cross-resistance and low fitness costs may explain why acquired antibiotic resistance can reach habitats such as drinking water.

Intersections between the water and the human-associated microbiome

Increasing evidences on the diversity, metabolic and functional capabilities of the microbiota associated with the human body show that microbial consortia play important roles in disease and health conditions, although their roles are not yet completely understood (Eloe-Fadrosh & Rasko, 2013; Turnbaugh *et al.*, 2007). Microorganisms colonizing or infecting humans may derive from different primary habitats, and not only the human body, and play distinct roles in health or disease status. The expression 'human-associated microbiome' is herein used to refer to all microorganisms capable of colonizing or infecting a human host independently of which is their primary habitat.

Two types of intersection between the human-associated microbiome and water habitats are expected. One refers to the release of bacteria from humans to wastewater. The other comprises bacteria that being present in drinking water are also reported in the human-associated microbiome. The first type of intersection was comprehensively analyzed by McLellan *et al.* (2010) who concluded that, as expected, only a small fraction of bacteria excreted by humans were represented in sewage and even less were found in surface water. Among the bacterial lineages found throughout these compartments, the predominant were *Lachnospiraceae*, *Bacteroidaceae* and *Ruminococcaceae* (McLellan *et al.*, 2010), groups poorly characterized in terms of antibiotic resistance. Other intersections are widely known as those of the indicators *E. coli* and enterococci, which representativeness in water and human-associated microbiomes is not so evident as could be expected (Table S1) (Qin *et al.*, 2010; Arumugam *et al.*, 2011; The Human Microbiome Project Consortium, 2012).

The assessment of the second type of intersection is even more difficult. The occurrence of antibiotic resistant bacteria in drinking water may be important because of the harmful effects that this could have in the human health. In such case, transmission could be directly of water bacteria to humans or, indirectly, via transmission of resistance genes from water bacteria to human-related bacteria (Fig. 3). Lee *et al.* (2010) used germ-free mice to demonstrate a correlation between the microbiota of drinking water and its presence in the gastrointestinal tract. However, this approach hardly can be used to infer about the fate of antibiotic resistant bacteria in the human gastrointestinal tract, given the richness and diversity of such habitat. Considering the value of taxonomy and phylogeny in the prediction of the ecology and physiology of bacteria, the currently available information about human and environmental microbiomes may allow interesting inferences. Using this rationale, the occurrence of the same

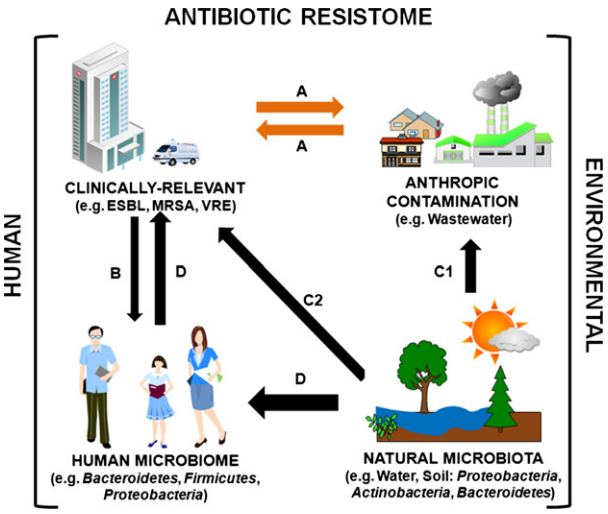

Fig. 3. Hypothesis about the relationship between environmental and human antibiotic resistome. (A) cycle of known clinically relevant antibiotic resistance determinants; (B) transfer of antibiotic resistance genetic determinants from clinically relevant bacteria to commensal human microbiota; (C) transfer of antibiotic resistance genetic determinants from the natural resistome to clinically relevant bacteria either thriving in the environment (C1) or hosted by humans (C2); (D) indirect transfer of antibiotic resistance determinants from the natural resistome to clinically relevant bacteria via human microbiome.

bacterial lineages in drinking water and in the human-associated microbiome may be an indication of the fitness of those bacteria to the human body. In addition, it may suggest its potential to, under favorable conditions, e.g. antibiotherapy, suffer positive selection or promote horizontal gene transfer. The search of bacterial groups found in water habitats (Table S1) in the NIH Human Microbiome Project catalog (http://www.hmpdacc.org/catalog/) revealed that 35 groups, distributed by five phyla (*Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Cyanobacteria*), found in treated drinking water can also be detected in the human-associated microbiome (e.g. in the gastrointestinal tract, oral cavity or skin, including lesions). Identically, 19 lineages distributed by three phyla (*Proteobacteria*, *Actinobacteria* and *Firmicutes*), found in mineral water can also be found in the human-associated microbiome (Table S1; Fig. 1). Probably, in the future, when more data are made available, more bacterial groups will be observed to be common to water environments and the human body. Nevertheless, it is already worthy of note that bacteria of the genera *Burkholderia*, *Acinetobacter*, *Aeromonas*, *Klebsiella*, *Pseudomonas*, *Stenotrophomonas* or *Clostridium* (Table S1), all of them with high potential to acquire antibiotic resistance genes (Zhang *et al.*, 2009), can be found in drinking water and in the human-associated microbiome. Others such as members of the genera *Sphingomonas* or *Methylobacterium* which exhibit resistance to several antibi-

otics, but about which almost nothing is known about antibiotic resistance genetics (Furuhata *et al.*, 2006, 2007; Vaz-Moreira *et al.*, 2011b), can also be found in water habitats and in the human-associated microbiome. The meaning of these evidences is still unclear but it may hint a link between water habitats and the human body, giving support to the hypothesis that water habitats may, directly or indirectly, supply antibiotic resistant bacteria for the human-associated microbiome (Fig. 3).

Missing links between natural and contaminant antibiotic resistance

Water and soil are regarded as important potential antibiotic resistance reservoirs, either natural or due to animal (and manure used as fertilizer) and human derived environmental contamination (Bush *et al.*, 2011; Forsberg *et al.*, 2012). However, except in a few well documented cases (e.g. *qnr* and $bla_{\text{CTX-M}}$) (Poirel *et al.*, 2002, 2005), it is difficult to demonstrate the passage of resistance genes from the environment to clinically-relevant bacteria or to clarify the mechanisms that made such a gene transfer possible. Previous studies have demonstrated that the human gut antibiotic resistome comprises an impressive myriad of antibiotic resistance genes not identified before and evolutionarily distant from the currently known resistance genes (Sommer *et al.*, 2009). The increasing number of complete bacterial genome sequences, support this observation (http://www.ncbi.nlm.nih.gov/genome). Putative annotation data, available in public databases, suggest that multidrug resistance as well as other specific resistance mechanisms are widespread in *Bacteria*. However, the annotated function encoded by these genome sequences is not reliable to infer with accuracy the expected phenotypes, mainly because the phenotype encoded by a gene may depend on the genetic and physiological environment (e.g. silent resistance genes) (Dantas & Sommer, 2012). Probably, most of the still unknown resistome is composed by resistance genes not yet validly annotated and others which expression is host-dependent. However, the clinical relevance of these genetic determinants as well as their influence on antibiotic resistance emergence is not clear yet. Although it can be hypothesized that the 'unkown' human resistant microbiome may represent the missing link between the environment and the human pathogens, evidences that ingested products (food and water) can be the major sources of antibiotic resistance genes are still missing.

Antibiotic therapy imposes profound and long lasting rearrangements in the human-associated microbiome, characterized by the increase of *Proteobacteria* and the simultaneous reduction of other groups such as *Bacteroidetes* or *Firmicutes* (Antonopoulos *et al.*, 2009; Jakobsson *et al.*, 2010; Jernberg *et al.*, 2010; Young & Schmidt, 2004). Eventually, it can be argued that, under specific conditions (e.g. antibiotherapy), minor or silenced parts of the human antibiotic resistome may lead important microbial and genomic rearrangements responsible for resistance development. Apparently, the environmental and pathogenic resistomes are not distinct, with the same genes being detected in both, although with higher prevalence in the pathogenic resistome (D'Costa *et al.*, 2006; Allen *et al.*, 2010; Forsberg *et al.*, 2012) (Fig. 3). Indeed, antibiotic resistance genes and gene mobilization cassettes, many of which without recognized clinical relevance, are widespread in nature, spanning numerous lineages of the bacterial world (Allen *et al.*, 2010; Cantón, 2009). However, apparently only a small fraction of these genetic elements was successfully spread through animals, humans and the environment, representing a public health threat. Which are the genetic characteristics or the external conditions that support the evolutionary success of an antibiotic resistance gene is still a major question.

Concluding remarks

In summary, the previous discussion on the diversity and ecology of water bacteria and antibiotic resistance led to a few conclusions and raised some new hypothesis:

1 Water habitats host an impressive bacterial diversity. However, only a few lineages are known to harbor antibiotic resistance genes of already recognized clinical relevance. The hypothesis that many bacterial lineages, some of them still unculturable, inhabiting water may represent a reservoir of new or emerging antibiotic resistance determinants cannot be discarded;

2 Bacteria belonging to the same bacterial lineages inhabit different types of water, including pristine water, disinfected water and raw wastewater. The hypothesis that these lineages can transfer relevant properties, mainly those that can be acquired by horizontal gene transfer, from unclean water habitats to clean environments, cannot be discarded;

3 Only a few groups of bacteria found in waters were, so far, identified in the human-associated microbiome. Although it is still uncertain in which cases the same species and strain can live in water and colonize humans, it is arguable that at least some of those lineages can represent a link between the water habitats and humans. In such case, those bacteria may be involved in the direct or indirect transfer of properties, including antibiotic resistance;

4 Well-known human commensal (as coliforms or enterococci) and pathogenic bacteria are minor and often undetected representatives of the water microbial com-

munities assessed based on metagenomic analysis. Therefore, metagenomic approaches may be of limited value to detect antibiotic resistance determinants already described in these organisms, unless enrichment or targeted methods are used.

5 Studies designed to survey the phylogeny of the antibiotic resistance genes and tracking the same gene types over different environmental compartments may contribute to shade some light on the relevance of environmental bacteria on the spread and transfer to humans of antibiotic resistance.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Bacterial diversity observed in different types of water, presence in the human-associated microbiome and occurrence of antibiotic resistance genes already characterized.

Table S2. Examples of antibiotic resistance genes detected in surface water, drinking water and wastewater.